PCT/AU2004/001630

Australian Government

Patent Office
Canberra

I, LEANNE MYNOTT, MANAGER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003906813 for a patent by EPITAN LIMITED as filed on 24 November 2003.

WITNESS my hand this
Seventh day of December 2004

LEANNE MYNOTT
MANAGER EXAMINATION SUPPORT
AND SALES

Regulation 3.

EpiTan Limited

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Method of Inducing Melanogenesis in Humans"

The invention is described in the following statement:

METHOD OF INDUCING MELANOGENESIS IN HUMANS.

FIELD OF THE INVENTION

The present invention relates broadly to a method of inducing melanogenesis in humans, that is stimulating the production of melanins by the pigment-producing cells (keratinocytes and/or melanocytes) of the skin, in particular in humans having a loss-of-function variations or mutations in the melanocortin-1-receptor gene.

BACKGROUND OF THE INVENTION

The melanocortins include a family of peptide hormones that induce pigmentation by interaction with melanocortin 1 receptors (MC1R) in the epidermis[1]. The primary pigmentary hormone that is released from the pars intermedia of the pituitary gland in some non-human animals, and from UV-B exposed keratinocytes in human skin, is alpha melanocyte stimulating hormone (alpha-MSH)[1]. This 13 amino acid peptide binds to MC1R to induce cyclic AMP-mediated signal transduction leading to the synthesis of melanin polymers from DOPA precursors[1]. Two type of melanins can be expressed in humans. The brownish - black pigment eumelanin is believed to convey protection from sun damage, whereas the reddish, sulfur-containing pigment, pheomelanin is often expressed in light-skinned human populations that report a poor tanning response to sunlight[1]. These poorly-tanning, easily-burning populations, often possess defects in the MC1R gene[2], and are generally thought to be at a greater risk of developing both melanoma and non-melanoma skin cancers[3,4].

Investigations conducted into human MC1R variants or mutations have established that "loss-of-function" mutations in the MC1R gene sensitise human melanocytes to the DNA damaging effects of UV radiation, which may increase skin cancer[5]. In addition, it has been demonstrated that human melanocytes having such "loss-of-function" mutations in the MC1R gene, such as R160W, D294H and R151C substitutions, demonstrate a significantly reduced response to alpha-MSH[5]. Four MC1R variant alleles, D84E, R151C, R160W and D294H, are reported to be strongly associated with red hair and fair skin in humans, characterised by a low melanin content and a low eumelanin to

pheomelanin ratio[6], and three of these alleles (R151C, R160W and D294H) have been shown to have an increased risk of cutaneous malignant melanoma (CMM)[4].

It has previously been disclosed that a super-potent derivative of alpha-MSH, melanotan-1, (Nle[4]-D-Phe[7]-alpha MSH), can induce tanning in human volunteers[7]. Melanotan (MT-1), contains two amino acid substitutions and is approximately 100 to 1,000-fold more potent than the native hormone at inducing pigmentation in experimental systems such as the frog skin bioassay[8] or in cultured human keratinocytes[8]. In humans, MT-1 primarily induces eumelanin synthesis in the skin in concert with it's tanning effect[9]. Although melanotropins have been postulated to effect immunologic changes[10-12], all of the prior trials reported only minimal side effects such as facial flushing and transient GI upset, unless doses greater than those needed for tanning were administered[13].

US Patent No. 4,457,864 (issued July 3, 1984), discloses analogues of alpha-MSH, including Nle[4]-D-Phe[7]-alpha MSH. Cyclic analogues of alpha-MSH are disclosed in US Patent No. 4,485,039 (issued November 27, 1984). The use of these and other analogues of alpha-MSH for stimulating the production of melanin by integumental melanocytes is disclosed in Australian Patent No. 597630 (dated January 23, 1987) and US Patents Nos. 4,866,038 (issued September 12, 1989), 4,918,055 (issued April 17, 1990) and 5,049,547 (issued September 17, 1991). Australian Patent No. 618733 (dated May 20, 1988), and US Patents Nos. 5,674,839 (issued October 7, 1997), 5,683,981 (issued November 4, 1997) and 5,714,576 (issued February 3, 1998) disclose further linear and cyclic alpha-MSH fragment analogues, and the use of these biologically-active analogues in stimulating melanocytes. The contents of all these published Australian and US patents are incorporated herein by reference.

In work leading to the present invention, it has been demonstrated that notwithstanding the significantly reduced response to alpha-MSH of human melanocytes having "loss-of-function" variations or mutations[5], MT-1 is effective in inducing melanogenesis in human subjects having MC1R variant alleles. In particular, it has been demonstrated that significant increases in melanin density can be induced in such subjects by use of MT-1, in some cases leading to melanin density levels similar to the levels in subjects having the wild-type MC1R.

Accordingly, the method of the present invention enables the induction of melanogenesis in human subjects having a "loss-of-function" variation or mutation in the MC1R gene, leading to increased

melanin density levels in these subjects and reduced risk of skin cancer.

SUMMARY OF THE INVENTION

Bibliographic details of the publications referred to in this specification by reference number are collected at the end of the specification.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications, the invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

In one aspect, the present invention provides a method for inducing melanogenesis in a human subject having an MC1R variant allele associated with loss of receptor function, which comprises the step of administering to said subject an amount of an alpha-MSH analogue effective to induce melanogenesis by the melanocytes in the skin or other epidermal tissue of the subject.

DETAILED DESCRIPTION OF THE INVENTION

As described above, the present invention provides a method for inducing melanogenesis in a human subject having an MC1R variant allele associated with loss of receptor function, which comprises the step of administering to said subject an amount of an alpha-MSH analogue effective to induce melanogenesis by the melanocytes in the skin or other epidermal tissue of the subject.

Human subjects having an MC1R variant allele associated with loss of receptor function, demonstrated by a reduced response to alpha-MSH, have a so-called "loss-of-function" variation or mutation[5] in the MC1R gene. The melanocytes of such subjects may be either homozygous or

heterozygous for such variations or mutations, and the loss of receptor function associated with the variation or mutation may vary from full to only partial loss of function. Particular variant alleles which are relevant to the method of the present invention include, by way of example, V60L, D84E, V92M, R151C, R160W, R163Q and N294H. The present invention extends to induction of melanogenesis in human subjects having one or more of these full or partial "loss-of-function" MC1R variant alleles.

Alpha-MSH analogues suitable for use in the method of the present invention include those disclosed in US Patents Nos. 4,457,864, 4,485,039, 4,866,038, 4,918,055, 5,049,547, 5,674,839, 5,683,981 and 5,714,576 and Australian Patents Nos. 597630 and 618733, and the disclosure of each of these patent documents is incorporated herein by reference.

In its broadest aspects, the present invention extends to the use of any of these alpha-MSH analogues. These analogues may be synthesised according to the procedures set out in these patent documents, or according to methods used in preparing synthetic alpha-MSH which are well-known to persons skilled in this art.

Suitable alpha-MSH analogues for use in accordance with the present invention include compounds of the formula:

R_1-W-X-Y-Z-R_2

wherein

R_1 is selected from the group consisting of Ac-Gly-, Ac-Met-Glu, Ac-Nle-Glu-, and Ac-Tyr-Glu-;

W is selected from the group consisting of –His- and –D-His-;

X is selected from the group consisting of –Phe-, -D-Phe-, -Tyr-, -D-Tyr-, -(pNO_2)D-Phe^7-;

Y is selected from the group consisting of –Arg- and –D-Arg-;

Z is selected from the group consisting of –Trp- and –D-Trp-; and

R_2 is selected from the group consisting of –NH_2; -Gly-NH_2; and –Gly-Lys-NH_2.

As used hereinabove and below, Ala = alanine, Arg = arginine, Dab = 2,4 diaminobutyric acid, Dpr = 2,4 diaminopropionic acid, Glu = glutamic acid, Gly = glycine, His = histidine, Lys = lysine, Met = methionine, Nle = norleucine, Orn = ornithine, Phe = phenylalanine, (pNO_2)Phe = paranitrophenylalanine, Plg = phenylglycine, Pro = proline, Ser = serine, Trp = tryptophan, TrpFor =

N^{1}- formyl-tryptophan, Tyr = tyrosine, Val = valine. All peptides are written with the acyl-terminal end at the left and the amino terminal end to the right; the prefix "D" before an amino acid designates the D-isomer configuration, and unless specifically designated otherwise, all amino acids are in the L-isomer configuration.

Compounds suitable for use in the present invention include:

[D-Phe^{7}]-alpha-MSH
[Nle^{4}, D-Phe^{7}]-alpha-MSH
[D-Ser^{1}, D-Phe^{7}]-alpha-MSH
[D-Tyr^{2}, D-Phe^{7}]-alpha-MSH
[D-Ser^{3}, D-Phe^{7}]-alpha-MSH
[D-Met^{4}, D-Phe^{7}]-alpha-MSH
[D-Glu^{5}, D-Phe^{7}]-alpha-MSH
[D-His^{6}, D-Phe^{7}]-alpha-MSH
[D-Phe^{7}, D-Arg^{8}]-alpha-MSH
[D-Phe^{7}, D-Trp^{9}]-alpha-MSH
[D-Phe^{7}, D-Lys^{11}]-alpha-MSH
[D-Phe-^{7}, D-Pro^{12}]-alpha-MSH
[D-Phe^{7}, D-Val^{13}]-alpha-MSH
[D-Ser^{1}, Nle^{4}, D-Phe^{7}]-alpha-MSH
[D-Tyr^{2}, Nle^{4}, D-Phe^{7}]-alpha-MSH
[D-Ser^{3}, Nle^{4}, D-Phe^{7}]-alpha-MSH
[Nle^{4}, D-Glu^{5},D-Phe^{7}]-alpha-MSH
[Nle^{4}, D-His^{6}, D-Phe^{7}]-alpha-MSH
[Nle^{4}, D-Phe^{7}, D-Arg^{8}]-alpha-MSH
[Nle^{4}, D-Phe^{7}, D-Trp^{9}]-alpha-MSH
[Nle^{4}, D-Phe^{7}, D-Lys^{11}]-alpha-MSH
[Nle^{4}, D-Phe^{7}, D-Pro^{12}]-alpha-MSH
[Nle^{4}, D-Phe^{7}, D-Val^{13}]-alpha-MSH
[Cys^{4}, Cys^{10}]-alpha-MSH
[Cys^{4}, D-Phe^{7}, Cys^{10}]-alpha-MSH
[Cys^{4}, Cys^{11}]-alpha-MSH

[Cys^5, Cys^{10}]-alpha-MSH

[Cys^5, Cys^{11}]-alpha-MSH

[Cys^4, Cys^{10}]-alpha-MSH$_{4-13}$

[Cys^4, Cys^{10}]-alpha-MSH$_{4-12}$

[Nle^4, D-Phe^7]-alpha-MSH$_{4-10}$

[Nle^4, D-Phe^7]-alpha-MSH$_{4-11}$

[D-Phe^7]-alpha-MSH$_{5-11}$

[Nle^4, D-Tyr^7]-alpha-MSH$_{4-11}$

[(pNO$_2$)D-Phe^7]-alpha-MSH$_{4-11}$

[Tyr^4, D-Phe^7]-alpha-MSH$_{4-10}$

[Tyr^4, D-Phe^7]-alpha-MSH$_{4-11}$

[Nle^4]-alpha-MSH$_{4-11}$

[Nle^4, (pNO$_2$)D-Phe^7]-alpha-MSH$_{4-11}$

[Nle^4, D-His^6]-alpha-MSH$_{4-11}$

[Nle^4, D-His^6, D-Phe^7]-alpha-MSH$_{4-11}$

[Nle^4, D-Arg^8]-alpha-MSH$_{4-11}$

[Nle^4, D-Trp^9]-alpha-MSH$_{4-11}$

[Nle^4, D-Phe^7, D-Trp^9]alpha-MSH$_{4-11}$

[Nle^4, D-Phe^7]-alpha-MSH$_{4-9}$

[Nle^4, D-Phe^7, D-Trp^9]-alpha-MSH$_{4-9}$

[Nle^4, Glu^5, D-Phe^7, Lys^{10}, Gly^{11}]-alpha-MSH$_{4-13}$

[Nle^4, Glu^5, D-Phe^7, Lys^{10}]-alpha-MSH$_{4-10}$

[Nle^4, Asp^5, D-Phe^7, Lys^{10}]-alpha-MSH$_{4-10}$

[Nle^4, Asp^5, D-Phe^7, Orn^{10}]-alpha-MSH$_{4-10}$

[Nle^4, Asp^5, D-Phe^7, Dab^{10}]-alpha-MSH$_{4-10}$

[Nle^4, Asp^5, D-Phe^7, Dpr^{10}]-alpha-MSH$_{4-10}$

[Nle^4, Asp^5, D-Phe^7, Lys^{10}, Gly^{11}]-alpha-MSH

[Nle^4, Asp^5, D-Phe^7, Lys^{10}]-alpha-MSH$_{1-10}$

[Nle^4, Asp^5, D-Phe^7, Lys^{10}]-alpha-MSH$_{2-10}$

[Nle^4, Asp^5, D-Phe^7, Lys^{10}]-alpha-MSH$_{3-10}$

[Nle^4, Asp^5, D-Phe^7, Lys^{10}]-alpha-MSH$_{4-10}$

[Nle^4, Asp^5, D-Phe^7, Lys^{10}, Gly^{11}]-alpha-MSH$_{4-11}$

[Nle^4, Asp^5, D-Phe^7, Lys^{10}, Gly^{11}]-alpha-MSH$_{4-12}$

[Nle^4, Asp^5, D-Phe^7, Lys^{10}, Gly^{11}]-alpha-MSH$_{4-13}$

[Nle^4, Asp^5, D-Phe^7, Lys^{10}, Gly^{11}]-alpha-MSH$_{3-13}$

Preferred compounds include:

[Nle^4, D-Phe^7]-alpha-MSH

[Nle^4, D-Phe^7]-alpha-MSH$_{4-10}$

[Nle^4, D-Phe^7]-alpha-MSH$_{4-11}$

[Nle^4, D-Phe^7, D-Trp^9]-alpha-MSH$_{4-11}$

[Nle^4, D-Phe^7]-alpha-MSH$_{4-9}$

The most preferred alpha-MSH analogue for use in the methods of this invention is [Nle^4, D-Phe^7]-alpha-MSH, referred to hereinafter as "melanotan-1" or "MT-1".

The alpha-MSH analogues used in the method of this invention may be administered by a variety of routes including oral, parenteral or transdermal. The term "parenteral" is used herein to encompass any method by which the compounds according to the present invention are introduced into the systemic circulation and include intravenous, intramuscular and subcutaneous injections. The term "transdermal" as used herein encompasses the administration of the compound by topical methods such as buccal or skin patches, intranasal or tracheal sprays, by solution for use as ocular drops, by suppositories for vaginal or anal routes of administration or by conventional topical preparations such as creams or gels for localised percutaneous delivery.

The compounds will be formulated in suitable compositions determined by the intended means of

administration, according to methods and procedures well-known to those skilled in the art (see, for example, *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Company, Pennsylvania, USA). For example, the compounds suitable for use in this invention may be formulated or compounded into pharmaceutical compositions comprising at least one compound of the present invention (the compositions may comprise one compound or admixtures of compounds according to the present invention) in admixture with a solid or liquid pharmaceutical excipient such as a diluent or carrier for oral or parenteral administration. As injection medium, water containing the usual pharmaceutical additives for injection solutions, such as stabilising agents, solubilising agents, and buffers is preferred. Among additives of this type are, for example, tartrate and citrate buffers, ethanol, complex forming agents such as ethylenediamine-tetraacetic acid, and high molecular weight polymers such as liquid polyethylene oxide for viscosity regulation. Solid carrier materials include, for example, starch, lactose, mannitol, methyl cellulose, talc, highly dispersed silicic acid, high molecular weight fatty acids such as stearic acid, gelatine, agar-agar, calcium phosphate, magnesium stearate, animal and vegetable fats, and high molecular weight polymers such as polyethylene glycols. Compositions suitable for oral administration can, if desired, contain flavouring and/or sweetening agents. For topical administration, the compounds may be preferably used with various conventional bases for topical preparations such as creams, ointments, gels, lotions or sprays, depending upon the desired mode of delivery of the ingredients to an individual. In manufacturing these preparations, the composition may also be mixed with conventional inert excipients such as thickening agents, emollients, surfactants, pigments, perfumes, preservatives, fillers and emulsifiers, all of which are well known and conventionally used in the formulation of transdermal or other preparations. Typically, these non-active ingredients will make up the greater part of the final preparation. Preferably, the compositions are manufactured to allow for controlled and/or sustained-release delivery.

The actual amount of administered compound according to the present invention may vary between fairly wide ranges depending upon the mode of administration, the excipients used, and the degree of stimulation desired. Such amounts are well within the skill of the pharmaceutical scientist to determine, and the amount administered to the mammal may be any amount chosen to stimulate melanotropic activity, for example, by formulation as an implant using poly (D, L lactide-co-glycolide polymer[14] or a similar biodegradable, biocompatible polymer as carrier.

In the work leading to the present invention, a double-blind, randomized, placebo-controlled clinical trial has been conducted with 81 human subjects, later reduced to 79 subjects. Melanotan (MT-1) was administered at a fixed, subcutaneous daily dose to 59 subjects, and the remaining 20 subjects were administered the placebo (saline). Fourteen subjects did not complete the trial protocol (twelve Melanotan and two placebo). Of the 47 subjects that were administered Melanotan and completed the trial protocol, 14 had no MC1R variant alleles while 33 had variant alleles. Average baseline melanin density (MD) and MD change (%) was measured in all 47 protocol completers. Subjects having the MC1R variant alleles V60L, V92M, R151C, R160W and R163Q had lower average baseline MD than the subjects having wild-type MC1R, however the MD change (%) over the trial period for subjects having MC1R variant alleles was significantly higher than the MD change (%) for subjects having wild-type MC1R, clearly and unexpectedly demonstrating the induction of melanogenesis in the subjects having MC1R variant alleles.

The present invention is further described by reference to the following non-limiting Example.

EXAMPLE

INTRODUCTION AND STUDY RATIONALE

There is compelling evidence that melanotropic peptides may provide a potential for increasing melanin pigmentation of human skin. Synthetic MSH may be used to enhance skin pigmentation of normal or light-skinned individuals to protect them from the hazards of solar radiation. Several studies have suggested that individuals whose skin tends to burn easily on exposure to the sun and does not tan readily are at higher risk of both nonmelanoma skin tumours and of cutaneous melanoma[15-17]. There is unambiguous evidence that UV radiation is responsible for skin cancer in humans. In the face of increased deterioration of the ozone layer, especially in Australia, and the increasing incidence of and mortality from skin cancer, the ability to stimulate the skin's own "protective mechanism" of tanning may prove extremely important as photoprotective strategy.

Several derivatives of α-MSH have been synthetised[18,19]. [Nle^4-D-Phe^7]- α-MSH, (Melanotan; MT-1); is the medication involved in this study. The substitution of amino acids at positions 4 and 7 makes this analogue 10-1000 times more active than α-MSH in one or more bioassays[19]. The pharmacological action of Melanotan is quite prolonged as evidenced by sustained maximal tyrosinase stimulation in cultured mouse melanoma cells[20]. The prolonged activity may result partially from its resistance to degradation by serum enzymes or proteolytic enzymes. These results

have very important implications for the use of this analogue in the therapeutic darkening of the skin to protect it from UV light.

Intraperitoneal delivery of Melanotan to foetal rats and mice also had no teratogenic or toxicological effects[21]. In addition, standard toxicology tests on mice, rats, guinea pigs and miniature pigs using subcutaneous and intraperitoneal injection routes have shown no significant toxicities[22,23,24] Several studies on human volunteers have been performed under a Physician's IND in the U.S. These studies, in over 100 subjects, have clearly demonstrated that Melanotan can induce safe and effective tanning in humans over a dose range of 0.08 to 0.4mg/Kg/day for 10 days[25,26,27] with follow-up to 12 months. Pharmacokinetic data has indicated that Melanotan has a half-life after subcutaneous administration of about 30 minutes with little or no activity in the plasma after 6 hours. There is no accumulation of Melanotan in subjects[28].

STUDY OBJECTIVES

Primary Safety Objective

To establish the safety and tolerability [defined as absence of any toxicities ≥ Grade 3 by WHO - CTC] of 3 (10 day; 5 days a week x 2 weeks) monthly courses of Melanotan (MT-1) at a fixed subcutaneous dose of 0.16mg/Kg/day in Caucasian subjects (skin types Fitzpatrick, I to IV[15]).

Primary Efficacy Objective

Degree of tanning.

To compare the degree of tanning at 8 anatomic sites (determined by serial reflectance changes) at Visit 33, 90 days after initiation of dosing with Melanotan and placebo in Caucasian subjects (skin type Fitzpatrick, I to IV[15]).

Secondary Efficacy Objective

Comparison of tanning in skin types.

To compare the degree of tanning at 8 anatomic sites determined by serial reflectance measurements performed at baseline and 30, 60, and 90 days after initiation of dosing in skin types Fitzpatrick I/II versus III/IV.

INVESTIGATIONAL PLAN

1. Selection of Study Population

The target population consisted of male and female Caucasian subjects. Seventy nine subjects were enrolled. Subject accrual was stratified to balance skin types with the intention of recruiting 40 subjects with skin types I/II and 40 subjects with skin types III/IV according to the Fitzpatrick scale. Subjects had a blood sample taken at screening for MC1-R genotyping but the stratification for this study was on the basis of Fitzpatrick skin type (a reassessment of the data according to genotype was performed at the completion of the study). The following inclusion and exclusion criteria had to be met by each subject before enrolment in the study.

Inclusion Criteria

- Male and Female Caucasian Subjects (skin types I to IV on the Fitzpatrick scale[15].)
- Age 18 – 65 years
- Weight $\leq$ 85 Kg
- Free of significant abnormal findings as determined by medical history (including family history), physical examination, haematology, plasma biochemistry and vital signs (blood pressure, pulse rate) determined at screening;
- Written informed consent prior to the performance of any study-specific procedures.
- Willing to provide 4 skin biopsy specimens (via drill or blister technique).

2. Study Medication

2.1 Description of Study Medication

Melanotan was provided in single-use sterile 6mL vials each containing 16mg of Melanotan dissolved in 1mL sterile saline for injection. Placebo vials were identical and contained 1mL sterile saline for injection.

2.2 Method of Assigning Subjects to Treatment Group

A randomisation (code) list was prepared which assigned study medication to individual subject numbers such that Melanotan and placebo was randomised in the ratio 3:1. The subject numbers were subdivided into 2 strata, the subdivision was such that the 3:1 Melanotan to placebo ratio was maintained.

Subjects in this study were stratified to one of the two strata based on skin type according to the following characterization:

Stratification	Characterization of skin type
1	Fitzpatrick I –II
2	Fitzpatrick III –IV

65 subjects were enrolled into Stratification # 1 and 24 into # 2 respectively.

2.3 Dosage and Administration of Study Medication

Active: Melanotan was provided in single-use, sterile 6 mL vials each containing 16 mg (± 5%) of Melanotan in 1 mL sterile saline. A dose of 0.16 mg/kg/day was administered by subcutaneous injection to each subject receiving the Melanotan treatment, which is equivalent to a dose volume of 0.01 mL/kg/day.

Placebo: Placebo was provided as single-use, sterile 6 mL vials containing 1 mL sterile saline. A dose volume of 0.01 mL/kg/day was subcutaneously injected at each administration.

The treatments were injected subcutaneously, using a 25 gauge needle (16 mm length) and 1 mL syringe, to the abdomen each day for 5 days a week x 2 weeks. Each subject's body weight was determined at check-in at Visit 3 (Day 1), and the same weight was used for all dose calculations for the first 10 days treatment. Dosing began at visit 3 following confirmation of eligibility. The clinical investigator or designated trained nominees, applied all

treatments. This cycle of treatment was repeated at Visits 13-22 (Days 29 to 40) and Visits 23-32 (Days 57 to 66). The subject was reweighed at the start of each dosing period for calculation of dose. Drug was given as nearly as possible at the same time each day (+/- 4 hours).

2.4 Blinding

The study medication was packaged according to the randomisation (code) list and the only identification that would reveal the details on the vials was the subject number. The investigator was given a list of subject numbers allocated to their centre. To randomise a subject they selected the next available sequential subject from the appropriate strata number from the list and then requested medication for this subject number from the pharmacist.

2.5 Use of sunscreen products:

All subjects were advised to apply SPF 25+ sunscreen to exposed skin whenever they expected to be in the sun for prolonged periods of time. Normal daily activity did not require extra precautions.

3. Study Procedures

3.1 Schedule of Investigations and Data Collection

This was an outpatient study. Subjects attended the clinic for a total of 34 visits throughout the 3 month study period. Subjects had a three-to-four visit screening period (minimum of 3 days). This allowed for informed consent, baseline assessments to determine their MC1-R genotype, fulfilment of all inclusion/exclusion criteria, determination of MED (minimal erythema dose), receipt of controlled UV radiation at 3.0 times their MED, the collection of skin biopsy specimens and full blood sampling.

This was followed by 5 days per week for two weeks (Visits 3-12) for study drug administration. The next visit was at after one month (Day 29; Visit 13) for blood sampling and start of next 10 day treatment over two weeks (Visits 13-22). Skin reflectance measurements were repeated on days 12, 30, 40 and 60. After two months, the cycle was repeated for the last dosage of study drug (Visits 23 –32). The final two visits (visits 33 and 34) occurred at

the end of week 13 when a sixth and final skin reflectance reading and sunburn evaluations were performed.

3.2 MC1-R Genotype Assessment

Subjects had a separate 10 mL blood sample taken at 1st screening visit for assessment of their MC1R. A 10 mL blood sample was used to isolate genomic DNA using a Qiagen genomic DNA extraction kit (QIAamp DNA Blood Maxi Kit (Cat # 51192). Whole blood samples were collected directly into Qiagen buffer in 10 mL test-tubes and frozen. Once all samples had been collected from all participants , the samples were sent to Menzies Centre for Population Research, Hobart (Professor Terry Dwyer) for subsequent analysis.

DNA Extraction

Genomic DNA was isolated from peripheral blood leukocytes using the Nucleon Bacc3 DNA extraction kit (Amersham International). Briefly, 30mL of Reagent A was added to 10mL of whole blood and inverted for 4 mins. Samples were centrifuged 5 mins at 2400 rpm and 20° C. The supernatant was removed and 2mls of Reagent B added. Samples were transferred into a 15mL centrifuge tube and vortexed for 10 seconds. 500µl of sodium perchlorate was added and the tube inverted 7 times. 2mL of chloroform was added and the inversion step repeated. Finally, 300µl of resin was added and the samples spun 3 mins at 2400 rpm and 20° C. The top layer (containing the DNA) was transferred into a clean 15mL tube with two volumes of cold absolute ethanol and inverted gently. The DNA was then transferred to a 1.5mL screw cap tube with the addition of 1mL 70% ethanol and inverted gently. Samples were centrifuged for 1 min at 13000 rpm and the ethanol eluted. Samples were then left to dry for ~20 min before re-hydration with 120µl of TE buffer. DNA samples were stored at 4° C.

Detection of MC1R gene variants

The MC1R gene fragment was amplified in two overlapping fragments using a PC960C thermal cycler (Corbett Research, Sydney). 29 variants, deletions or insertions were encompassed within the gene fragment, but only 9 were used in analysis with the EpiTan trial data. The primer sequences were: 5'-tggacaggactatggctgtg-3' (MC1R-1F), 5'-tcttcagcacgctcttcat-3' (MC1R-1R), 5'-cttctacgcactgcgctacc-3' (MC1R-2F) and 5'-

gctttaagtgtgctgggcag-3' (MC1R-2R). For individual amplifications 20-50ng genomic DNA template was combined with 10mM Tris-HCl, pH8.3, 50mM KCl, 2.0mM $MgCl_2$, 1µM each of the four dNTPs, 0.8µM of each primer, and 2.5U Taq polymerase (Qiagen) in a 30µl reaction. Samples were denatured for 2 min at 94°C, and amplified using 30 cycles consisting of 30 s at 94°C, 30 s at 62°C, and 30 min at 72°C, followed by a final elongation step for 10 min at 72°C.

Individual PCR reactions were purified using an UltraClean PCR Clean-up Kit (MO BIO Laboratories, Inc.). Forward and reverse sequences were amplified using an Applied Biosystem Big Dye Terminator Cycle Sequencing Kit (Applied Biosystem). Individual sequencing reactions of 10 µl contained 10-30 ng DNA template, 3.2 pmol/µl sequencing primer (MC1R-1/2F or MC1R-1/2R), and 4 µl Terminator Ready Reaction Mix. Samples were amplified using 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Sequencing reactions were ethanol precipitated as described in the ABI User Manual. Briefly, 30 µl cold 95% molecular grade ethanol and 2.5 µl 125mM EDTA were added to each reaction and mixed thoroughly. After 15 min incubation at room temperature samples were centrifuged 20 min at 13000 rpm. The supernatant was carefully removed and 30 µl cold 70% molecular grade ethanol was added. Samples were centrifuged 5 min at 13000 rpm and the supernatant was again removed. The DNA sequencing pellets were dried at 95°C for 1 min and stored at -20°C until ready for sequencing. Pellets were resuspended in 15 µl Template Suppression Reagent, vortexed and briefly centrifuged. Samples were denatured at 95°C for 2 min and then loaded onto an ABI Prism 310 Genetic Analyser. Both forward and reverse sequences of both MC1R fragments were sequenced and analysed using Sequencher 4.1 software (Gene Codes Corporation).

3.3. Assessment of Efficacy Variables

Skin Reflectance - Degree of tanning & melanin density (MD)

Before treatment at Visit 2 (Baseline; Day –1) and at Visit 12 (Day 12), Visit 14 (Day 30), Visit 22 (Day 40), Visit 26 (Day 60) and Visit 34 (Day 90), subjects had their skin pigmentation measured by a non-invasive quantitative skin chromaticity (reflectance) reading. Reflectance by the skin of wavebands of light measured at 20-nm intervals in the wavelength range 400-700 nm was recorded using a Minolta 508i spectrophotometer at eight skin sites (forehead,

cheeks, neck, scapula, inner upper arm, forearm, abdomen and calf). The spectrophotometer was programmed to take three separate measurements at each site at each session to minimize error. A diagram for each subject was provided at baseline and measurements positions for all eight skin sites at baseline were recorded on this diagram. Subsequent repeat measurements were done with reference to the initial diagram to ensure they were taken as close as possible to the original measurement at each skin site.

At each visit the mean of the 3 separate measurements taken at each site for the reflectance values at 400 and 420nm were obtained and recorded. Using the measurement of reflectance at 420nm minus that at 400nm a reasonable prediction of the melanin content of the skin was obtained, as described by Dwyer et al,[29]. The equation used was:

$$MD = 100 \times (0.035307 + 0.009974(R_{420} - R_{400}))$$

where MD is an estimate of the percentage of the epidermis of the skin that contains melanin, R_{400} and R_{420} denote reflectance at 400nm and 420nm, respectively. These MD measurements were calculated at the analysis stage.

4. Statistical Methods

4.1 Statistical and Analytical Plans

The statistical analysis was carried out using SAS statistical software from SAS Institute Inc., Cary, NC (USA), or other suitable statistical software.

Continuous data were summarised by presenting the number of subjects, median, mean, standard deviation, minimum and maximum values. Categorical data were summarised by presenting the number and percentage in each category.

All statistical tests of significance were performed at the 5% level of significance and were 2-sided unless otherwise stated. As this was an exploratory phase II trial, no adjustment to significance levels were made to account for multiple comparisons.

4.2 Efficacy Assessment

Specification of Primary Efficacy Endpoint

The primary efficacy objective was to compare the degree of tanning attained 90 days after initiation of dosing with Melanotan or placebo in Caucasian subjects (skin types Fitzpatrick, I to IV). The primary endpoint was:-

- Change in tanning from baseline (Visit 2) to day 90 across 8 anatomic sites (forehead, cheeks, neck, scapula, inner upper arm, forearm, abdomen and calf) determined by melanin density (MD) from skin reflectance measurements [Dwyer *et al.* [29]; MD=100 x (0.035307+0.009974 (R420 – R400)].

The change from baseline was calculated as the difference between the Visit 34 and baseline value (Visit 34 – Visit 2) at each of the 8 sites individually. The mean change across all eight sites was then calculated.

If a subject discontinued prior to the end of the study the last available value following treatment with study medication was used to calculate the change and used in the intent-to-treat analysis. The last available value was be carried forward to calculate the change for the per-protocol analysis.

Specification of Secondary Efficacy Endpoint

The secondary efficacy endpoints were:

Comparison of Tanning

- Change in melanin density (MD) at 30, 60 and 90 days after study dose initiation determined by skin reflectance measurements of 8 anatomic sites (forehead, cheeks, neck, scapula, inner upper arm, forearm, abdomen and calf) [Dwyer *et al.* [29]; MD=100 x (0.035307+0.009974 (R420 – R400)].

MC1-R Genotype

Data for this parameter were analysed via a separate protocol. Genotype information was collected from all studies using Melanotan in order to build up a data base of "skin types" for subsequent re-analysis of the effectiveness of Melanotan based on MC1-R genotype rather than Fitzpatrick classification.

4.3 Analysis Plan

Subject Analysis Sets

All subjects enrolled in the study were included in summaries of subject disposition, but subjects who discontinued prior to randomisation were excluded from all other aspects of the analyses.

Populations for Efficacy

There were 2 populations for efficacy:

- Intent-to-Treat (ITT) population.
- Per Protocol population (PP).

To be eligible for inclusion in the ITT population, subjects had been randomised, had been administered at least one dose of trial medication and had at least one post-randomisation evaluation for the variable concerned.

The PP population was a sub-set of the ITT population. This subset restricted eligibility to those subjects who completed the study (or who did not complete the study but had an efficacy endpoint within the efficacy evaluable window). Their efficacy measures had been taken at appropriate times and under appropriate conditions and they had not violated or deviated from the protocol in any manner that may impact the efficacy assessments.

The main population upon which efficacy conclusions were based were the ITT population. If the PP subset contained less than 90% of the ITT population then analyses were also performed for the PP population to support the conclusions reached, otherwise analyses were only performed for the ITT population.

Handling of Missing and Incomplete Data

If an efficacy measurement was missing, then for the purposes of the ITT analyses, the last available post-treatment measurement was carried forward and used in place of the missing value.

RESULTS

The following Table 1 lists the response of the 47 Melanotan protocol completers in terms of the change in melanin density (MD) measured at the inner upper arm after 90 days. The inner upper arm generally denotes a person's constitutive skin melanin since environmental exposure appears to be least in this area.

From Table 1 it can be clearly seen that the change in melanin density (MD) for the subjects with MC1R variant alleles was approximately double that of the wildtype population (1.00% vs 0.49% respectively). The MD change which ranged from 0.85% for R151C to 1.27% for R163Q represented an approximately 35% increase in the baseline level of melanin (which was 2.3 to 3.0%) This compares to only a 13% increase in the wildtype population.

This unexpected result indicates that people with MC1 receptors associated with these variant alleles can produce skin melanin in response to stimulation with Melanotan. It is generally accepted that these receptors (with variant alleles) do not bind well to the natural hormone, alpha-MSH, and would not therefore be expected to respond to Melanotan.

These six variant alleles have been associated to varying degrees with an increased risk of skin cancer. Since all but one have been shown, in this study, to respond to Melanotan this could prove very beneficial as a skin protective agent for these people. More numbers need to be included for people with D294H variant allele. In this study of just one person with this variant allele there was no increase in melanin.

TABLE 1 Results of Melanin Density Changes (inner upper arm) in Various Alleles

(47 protocol completers for 90 days)

Variant Allele	No. of subjects	No. of alleles	Average Baseline MD [% of melanin in epidermis of skin]	MD Change %
Total	33**	51	2.88 ± 0.20	1.00 ± 0.12
Wildtype	14	0	3.63 ± 0.33	0.49 ± 0.11
Val60Leu (V60L)	17*	18	2.72 ± 0.33	1.18 ± 0.17
Val92Met (V92M)	11	11	3.01 ± 0.27	1.03 ± 0.13
Arg151Cys (R151C)	10	10	3.03 ± 0.42	0.85 ± 0.34
Arg160Trp (R160W)	4	4	3.10 ± 0.53	1.11 ± 0.52
Asp294His (D294H)	1	1	2.16	- 0.23
Arg163Gln (R163Q)	4	4	2.30 ± 1.05	1.27 ± 0.49

* 1 person was homozygous for this allele and therefore denotes 2 alleles.

** Thirty three subjects had 48 MC1R alleles (some individuals had two alleles)

Table 2 denotes the distribution of variant alleles in the total protocol population of 79 subjects. Eighteen of these individuals were on placebo and fourteen withdrew prior to completion of the study leaving 47 active subjects to complete.

Importantly, this Table indicates that in a "blinded" recruitment of individuals from two main cities (Adelaide and Sydney) almost 70% had variant alleles at the skin MC1 receptor. Equally, 70% were described as skin type I/II in this population (55/79). However, not all skin type I/II individuals had variant alleles. The relationship between the possession of a variant allele and designated skin type has yet to be resolved.

TABLE 2: MCIR Variant Allele Distribution In Subject Population

MC1R Variant allele	Total number in subject population [n=79]	Variant as % of total variant alleles [n=72]	Variant as % of subject population [n=79]
V60L	30	41.7	38.0
D84G	2	2.8	2.5
V92M	12	16.7	15.2
R142H	2	2.8	2.5
R151C	16	22.2	20.3
R160W	6	8.3	7.6
D294H	4	5.6	5.1
Total subjects with Alleles	54		68.4
Total subjects with None (wildtype)	25		31.6

References

1. Hadley ME. The melanotropic hormones. *In*: Brake D, editor. Endocrinology. 4th Edition, Simon & Schuster; (1982). p. 153-76.

2. Valverde P, *et al.*. Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. *Nature Genet* (1995);**11**:328-30.

3. Box NF. *et al.*. Melanocortin-1 receptor genotype is a risk factor for basal and squamous cell carcinoma. *J Invest Derm* (2001); **116**:224-29.

4. Palmer JS. *et al.* Melanocortin-1 receptor polymorphisms and risk of melanoma: is the association explained solely by pigmentation phenotype? *Am J Hum Genet* (2000); **66**:176-86.

5. Scott, CS, *et al.*. Human melanocortin-1 receptor variants, receptor functions and melanocyte response to UV radiation. *J. Cell Science* (2002); **116**:2349-56.

6. Sturm, RA, *et al.*. Genetic association and cellular function of MC1R variant alleles in human pigmentation. *Ann NY Acad. Sci.* (2003); **994**:348-58.

7. Levine N, *et al.* Induction of skin tanning by subcutaneous administration of a potent synthetic melanotropin. *JAMA* (1991); **266**:2730-736.

8. Sawyer TK, *et al.* [Nle^4-D-Phe^7)-α-melanocyte stimulating hormones: a highly potent α-melanotropin with ultralong biological activity. *Proc Natl Acad Sci* USA (1980); **77**:5754-8.

9. Dorr RT, *et al.* Increased eumelanin expression and tanning is induced by a superpotent melanotropin [Nle^4D-Phe^7]-α-MSH in humans. *Photochem Photobiol* (2000); **72**:526-32.

10. Lipton JM, *et al.* Antiinflammatory effects of the neuropeptide alpha-MSH in acute, chronic and systemic inflammation. *Ann NY Acad Sci* (1994); **741**:137-48.

11. Ceriani G, *et al.* The neuropeptide alpha-melanocyte-stimulating hormone inhibits experimental arthritis in rats. *Neuroimmunomodulation* (1994); **1**:28-32.

12. Chiao HS, *et al.* α-melanocyte-stimulating hormone reduces endotoxin-induced liver inflammation. *J Clin Invest* (1996); **97**:2038-44.

13. Levine N, *et al.* Effects of a potent synthetic melanotropin, Nle^4-D-Phe^7-α-MSH (Melanotan-1) on tanning: a dose-ranging study. *J Derm Treat* (1999); **19**:127-32.

14. Vardwaj R, Hadley ME, Dorr RT, Dvorakova K, Brooks C, Blanchard J. Pharmacologic response of a controlled-release PLGA formulation for the alpha-melanocyte stimulating hormone analog, Melanotan-1. *Pharmaceutical Research* (2000); 17:583-9.

15. Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. *Arch. Dermatol.* (1988) **124**: 869–871.

16. Graham JH. Precancerous lesions of the skin. *Primary Care* (1975) **2**: 699 –766.

17. Stenback F. Life history and histopathology of ultraviolet light-induced skin tumours. *National Cancer Institute Monograph* (1978) **50**: 57-70.

18. De L. Castrucci AM *et al.* Synthesis and studies of superpotent melanotropins resistant to enzyme degradation. *Comp. Biochem. Physiol.* (1984) **78B**: 519-524.

19. Sawyer TK, *et al.* 4-Norleucine, 7D-phenylalanine-α-melanocyte stimulating hormone: a highly potent -α-melanotropin with ultraprolonged biological activity. *Proc. Nat. Acad. Sci. USA* (1980) **77**: 5754 – 5758.

20. Hadley ME *et al.* [Nle^4-D-Phe^7]- α-MSH: a superpotent melanotropin that "irreversibly" activates melanoma tyrosinase. *Endocr. Res.* (1985) **11**: 157-170.

21. Dawson BV *et al.* Administration of melanotropic peptides during gestation in the rodent. *Toxicol.* (1993) **77**: 91-101.

22. Dorr RT *et al.* Toxicologic studies of a superpotent α- melanotropin, [Nle^4-D-Phe^7]- α-MSH. *Invest. New Drugs* (1988) **6**: 251-258.

23. Dorr RT. and Dawson BV. Toxicology report: Results of a 30 day study of MELANOTAN –I given subcutaneously to adult rats. *University of Arizona internal report.* (1988).

24. Dorr RT . Thirty day Toxicology study of Melanotan-I [Nle^4-D-Phe^7]- α-MSH in miniature Yucatan swine. *University of Arizona, Laboratory Booklet -Chris Brooks* (1993).

25. Levine N *et al.* Induction of skin tanning by subcutaneous administration of a potent synthetic melanotropin. *J. Am. Acad. Sci.* (1991) **266**: 2730 – 2736.

26. Levine N *et al.* Effects of a potent synthetic melanotropin, Nle^4-D-Phe^7- α-MSH (Melanotan-I) on tanning: Dose ranging study. *J. Dermatol. Treat.* (1999) **10** (2): 127-132.

27. Dorr RT *et al.* Increased eumelanin expression and tanning is induced by a superpotent melanotropin [Nle^4-D-Phe^7]- α-MSH in humans. *Photochem & Photobiol.* (2000).

28. Evans A *et al.* A Randomised, Placebo-Controlled, Double-Blind Study to Assess the Pharmacokinetics and Tanning Effect of Melanotan in Healthy Adult Subjects *Study Report August* (2000).

29. Dwyer T *et al.* The use of spectrophotometry to estimate melanin density in Caucasians. *Cancer Epidemiology, Biomarkers & Prevention* (1998) **7**: 203–206.

Dated this 24th day of November 2003.

EpiTan Limited
By its Patent Attorneys
Davies Collison Cave

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/AU04/001630

International filing date: 23 November 2004 (23.11.2004)

Document type: Certified copy of priority document

Document details: Country/Office: AU
Number: 2003906813
Filing date: 24 November 2003 (24.11.2003)

Date of receipt at the International Bureau: 13 December 2004 (13.12.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)